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Detection of Nonhemagglutinating Influenza A(H3) Viruses by Enzyme-Linked Immunosorbent Assay in Quantitative Influenza Virus Culture

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To assess the efficacy of novel antiviral drugs against influenza virus in clinical trials, it is necessary to quantify infectious virus titers in respiratory tract samples from patients. Typically, this is achieved by inoculating virus-susceptible cells with serial dilutions of clinical specimens and detecting the production of progeny virus by hemagglutination, since influenza viruses generally have the capacity to bind and agglutinate erythrocytes of various species through their hemagglutinin (HA). This readout method is no longer adequate, since an increasing number of currently circulating influenza A virus H3 subtype (A[H3]) viruses display a reduced capacity to agglutinate erythrocytes. Here, we report the magnitude of this problem by analyzing the frequency of HA-deficient A(H3) viruses detected in The Netherlands from 1999 to 2012. Furthermore, we report the development and validation of an alternative method for monitoring the production of progeny influenza virus in quantitative virus cultures, which is independent of the capacity to agglutinate erythrocytes. This method is based on the detection of viral nucleoprotein (NP) in virus culture plates by enzyme-linked immunosorbent assay (ELISA), and it produced results similar to those of the hemagglutination assay using strains with good HA activity, including A/Brisbane/059/07 (H1N1), A/Victoria/210/09 (H3N2), other seasonal A(H1N1), A(H1N1)pdm09, and the majority of A(H3) virus strains isolated in 2009. In contrast, many A(H3) viruses that have circulated since 2010 failed to display HA activity, and infectious virus titers were determined only by detecting NP. The virus culture ELISA described here will enable efficacy testing of new antiviral compounds in clinical trials during seasons in which nonhemagglutinating influenza A viruses circulate.

Efficacy testing of novel antiviral drugs generally involves measuring infectious virus titers in clinical samples at treatment baseline and during follow-up. For influenza virus, an assessment of virus titers by endpoint dilution assays commonly relies on the measurement of viral hemagglutination activity in culture supernatants to detect the production of progeny virus. However, the continuous accumulation of amino acid substitutions in the viral glycoprotein hemagglutinin (HA) has complicated the detection and characterization of antigenic properties of influenza A(H3N2) viruses by hemagglutination and hemagglutination inhibition (HI) assays, respectively. Poor replicative capacity in Madin-Darby canine kidney (MDCK) cells has been associated with low binding affinities of the viral HA molecules to their receptors (1, 2). Reduced receptor binding is also evidenced by a failure to agglutinate efficiently, if at all, erythrocytes of various species (2–6). Amino acid substitutions in or around the receptor binding site of the HA molecule of A(H3N2) influenza viruses affected the capacity of the virus to agglutinate red blood cells (RBCs) (2, 5, 6), and the receptor binding preference has evolved from short branched sialylated glycans to sialic acids on long polylactosamine chains (3).

The replicative capacity of some virus isolates improved upon serial passaging in MDCK cells (1) or MDCK cells that overexpressed SA(α2,6)Gal receptor (MDCK-SIAT) (7). This may aid in performing HI assays with these viruses to assess their antigenic properties, provided that these remain unaffected during passaging. However, despite additional passages, the frequency of influenza A virus H3 subtype (A[H3])isolates with impaired HA activity has increased in The Netherlands since 2010, as reported here. Moreover, serial passaging is not an option in clinical trials that require data on infectious virus titers in original clinical specimens. The number of PCR-confirmed samples containing influenza A(H3N2) virus that had a false-negative result in quantitative culture assays with HA activity as a readout has also increased since 2010, whereas such discordant results were not found for samples containing influenza A(H1N1) or B viruses (our unpublished data).

Thus, alternative methods are required to monitor the replication of currently circulating A(H3) viruses in quantitative virus cultures. Scoring the viral cytopathic effect (CPE) could be considered, but this procedure lacks specificity and is labor-intensive, subjective, and not suitable for high-throughput applications. Alternatively, viral antigen can be detected in cell culture by enzyme-linked immunosorbent assay (ELISA), which is compatible with standardized automated procedures. Although an ELISA has been developed for the measurement of residual virus replication in virus neutralization assays (8, 9), it has never been applied to the assessment of virus titers in clinical samples. Here, we report the development and validation of an ELISA for the detection of the viral nucleoprotein (NP) in standard 6-day quantitative influenza

Received 23 December 2013 Returned for modification 28 January 2014
Accepted 4 March 2014
Published ahead of print 12 March 2014
Editor: A. J. McAdam
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.03575-13.
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doi:10.1128/JCM.03575-13

A virus cultures. We investigated the kinetics of virus replication using the NP-ELISA method and compared the virus titers with those obtained with the conventional HA assay, using laboratory strains and low-passage virus isolates with or without good hemagglutinating activity. Finally, the performances of the assays for assessing infectious virus titers in clinical samples were compared, which indicated that for recent A(H3) influenza viruses, the NP-ELISA readout method is superior.

MATERIALS AND METHODS

Virus strains, virus isolates, and clinical specimens. The laboratory strains A/Brisbane/059/07 (H1N1) and A/Victoria/210/09 (H3N2) were obtained from the National Institute for Biological Standards and Control (NIBSC) (South Mimms, Potters Bar, United Kingdom). Epidemic viruses were isolated and propagated in MDCK cells at the National Influenza Centre, Erasmus MC, Rotterdam, The Netherlands, as described previously (10), and included the (sub)types A(H1N1), A(H1N1)pdm09, A(H3N2), and B viruses, as determined by PCR. One set of respiratory clinical specimens, consisting of nasal wash and sputum samples, was submitted for routine testing to determine the presence of respiratory viruses at the diagnostic unit of the Department of Viroscience of the Erasmus MC, Rotterdam, The Netherlands. Approval by the local medical ethics board was obtained before the experiments commenced. Informed consent was waived because patient inclusion was performed retrospectively and the data were anonymously stored as agreed by the Erasmus MC institutional review board (MEC-2012-599). A second set of clinical specimens, consisting of nasopharyngeal swabs, was submitted for the purpose of assessing virus titers as part of a clinical trial (registered at ClinicalTrials.gov under registration no. NCT00988325 [WP22849]), and appropriate informed consent and ethics approval were obtained.

Cells. Madin-Darby canine kidney (MDCK) cells were cultured in Eagle minimal essential medium (EMEM) (Lonza, Verviers, Belgium) containing 20 mM HEPES buffer (Lonza), 0.075% sodium bicarbonate solution (Lonza), 2 mM L-glutamine (Lonza), 100 IU/ml penicillin-100 μg/ml streptomycin (Lonza), referred to as complete medium (CM), supplemented with 10% fetal bovine serum (FBS) (Bodinco BV, Alkmaar, The Netherlands). The cells were passaged to new culture flasks twice weekly. One or two days before inoculation, the cells were seeded in flat-bottom 96-well tissue culture-treated microplates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands).

HA titer determination. Duplicate serial 2-fold dilutions of 50 μl MDCK culture supernatant in phosphate-buffered saline (PBS) were prepared before adding 50 μl of 0.5% turkey erythrocyte solution. Following 1 h incubation at 4°C, the hemagglutination patterns were scored. The reciprocal of the highest dilution showing full agglutination was taken as the HA titer.

PCR. Nucleic acids were extracted from 190 μl MDCK culture supernatant and eluted to a volume of 110 μl using a MagNA Pure LC (Roche Diagnostics, Almere, The Netherlands) or QIAsymphony (Qiagen, Venlo, The Netherlands). Before nucleic acid isolation, 10 μl phocine distemper virus (PDV) was added as an internal control. Cycle threshold (C_T) values and H1 and H3 subtypes were determined by real-time reverse transcription-PCR (RT-PCR) on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) or a LightCycler 480 system (Roche Diagnostics, Almere, The Netherlands), as described previously (11).

Virus titration. Confluent monolayers of MDCK cells were inoculated with replicate ($n = 4$) serial 10-fold dilutions of virus stocks or clinical samples in 96-well microtiter format in infection medium (IM), which was prepared by supplementing CM with 0.3% bovine serum albumin fraction V (Sigma-Aldrich), 2.5 μg/ml amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands), and trypsin (Lonza; the optimal concentration was determined for each lot). After 90 min at 37°C in a humidified 5% CO_2 incubator, the inocula were removed and the cells were washed with IM and cultured for 6 days in a humidified incubator at 37°C and 5% CO_2. Medium control wells ($n = 12$) were included on each plate, and at least two laboratory virus strains with predefined titers were included in each experiment for trending. The results were accepted when the titers of the trend control viruses were within predefined limits.

HA readout. The replication of influenza viruses in cell culture was monitored by measuring hemagglutination activity in the culture supernatants as follows: 25 μl undiluted supernatant was mixed with 50 μl PBS and 25 μl of a 1% turkey erythrocyte solution. After incubation for 1 h at 4°C, the hemagglutination patterns were scored, and the frequencies of positive and negative wells among the four replicates per dilution were used to calculate virus titers (log 50% tissue culture infective dose [$TCID_{50}$]/ml) according to the Spearman-Kärber method (12).

NP readout. The replication of influenza viruses was also assessed by measuring the production of NP by ELISA with the following procedure: at various time points postinoculation of the MDCK cells, the cell culture plates were washed once with Oxoid PBS (product no. BR0014G; Fisher Scientific, Landsmeer, The Netherlands), fixed by adding 200 μl acetone (80% in water) to each well, and stored at −20°C. After removal of acetone, the wells were washed trice with PBS-0.05% Tween 20 (Merck Millipore, Amsterdam, The Netherlands) before adding the broadly reactive (13, 14) influenza A virus NP-specific mouse monoclonal antibody HB65 (European Veterinary Laboratory [EVL], Woerden, The Netherlands). Approximately 100 ng of antibody HB65 was added per well in a volume of 200 μl PBS containing 2% skim milk powder (PBS-SMP) (product no. 70166; Sigma). After incubation for 1 h at room temperature, the plates were washed three times with PBS-0.05% Tween 20, and 3 ng of a horseradish peroxidase-labeled goat anti-mouse IgG antibody preparation (product no. 626520; Invitrogen) was added in 100 μl PBS-SMP and incubated for 1 h at room temperature. Following three wash steps with PBS-0.05% Tween 20, ready-to-use 3,3′,5,5′-tetramethylbenzidine (TMB) (product no. T0440; Sigma; 100 μl/well) was added, and the plates were incubated at room temperature for 30 min before adding 100 μl stop solution (1N H_2SO_4) (product no. 320501; Sigma). The optical densities at 450 and 620 nm were measured by using a Tecan F200 plate reader, and signal differences ($OD_{450-620}$) of ≥0.5 were considered positive and indicative of widespread virus replication. For lower levels of virus replication, e.g., in wells around the endpoint dilution, signals that were <0.5 but ≥0.2 were considered positive when the OD value exceeded 3-fold the mean plus 3 standard deviations (SD) of the uninfected cell control wells. Because signals of >0.5 also exceeded the latter threshold, the scoring of positive and negative wells for $TCID_{50}$ calculation was effectively based on the latter. The frequencies of positive and negative wells among the four replicates per dilution were used to calculate the virus titers (log $TCID_{50}$/ml) according to the Spearman-Kärber method (12).

RESULTS

Frequency of virus isolates with poor HA activity in The Netherlands. The annual frequencies of the influenza A(H3) virus isolates received at the National Influenza Centre (NIC) of The Netherlands between 1999 and 2012 that agglutinate turkey erythrocytes poorly (i.e., HA titers ≤ 2) are shown in Table 1. Frequencies increased from ~0% to 5% between 1999 and 2003 to ~12% to 26% between 2004 and 2008. The following two influenza seasons were dominated by A(H1N1)pdm09 viruses, and only a few A(H3N2) isolates were obtained, which all showed low HA activity. During the 2011-2012 season, the majority of isolates were of the A(H3) subtype, as determined by RT-PCR, and 50.9% ($n = 162$) had HA titers of ≤2. The isolates with higher HA titers showed no such temporal increase (data not shown).

To assess whether low HA activity in the 2011-2012 isolates was due to low virus levels or to poor capacity to agglutinate RBCs, we performed PCR, targeting the matrix gene segment, on the samples without HA activity ($n = 141$; one isolate was lost to follow-up) and on those with an HA titer of 2 ($n = 20$). As a control, we processed 10 isolates obtained during the same season with an HA

TABLE 1 Annual frequency of influenza A(H3) virus isolates with low HA titers

Influenza season (yr)	No. of A(H3) isolates	Results for HA titer of: 0		2		≤2	
		n	%	*n*	%	*n*	%
1999-2000	334	1	0.3	16	4.8	17	5.1
2000-2001	7		0.0		0.0		0.0
2001-2002	265		0.0		0.0		0.0
2002-2003	150	1	0.7		0.0	1	0.7
2003-2004	400		0.0	99	24.8	99	24.8
2004-2005	338	1	0.3	40	11.8	41	12.1
2005-2006	116		0.0	16	13.8	16	13.8
2006-2007	259	1	0.4	52	20.1	53	20.5
2007-2008	21	1	4.8	2	9.5	3	14.3
2008-2009	415	51	12.3	56	13.5	107	25.8
2009-2010	3	1	33.3	2	66.7	3	100.0
2010-2011	13	6	46.2		0.0	6	46.2
2011-2012	318	142	44.7	20	6.3	162	50.9
Total	2,639	205	7.8	303	11.5	508	19.2

titer of 4 and 10 isolates with an HA titer of 32, all in the same experiment. PCR signals indicated the presence of virus ($C_T < 40$) in two-thirds ($n = 94$ [67%]) of the 141 isolates without HA activity and in 11 (55%) of the 20 isolates with an HA titer of 2. The remaining cultures, with C_T values of >40, are likely to have been inoculated with specimens containing noninfectious virus and were excluded from further analyses with respect to HA activity.

PCR-positive isolates with high C_T values are likely to contain insufficient virus to obtain a positive HA result. These samples may represent viruses that have poor intrinsic *in vitro* replication capacities or that may not be viable for other reasons. The positive-control isolates with HA titers of 4 or 32 had median C_T values of 15.5 (range, 12.8 to 25.5) and 14.4 (range, 12.8 to 19.2), respectively. As expected, the C_T values were generally higher in isolates with HA titers of 2 (16.0 [range, 14.2 to 30.6]) or 0 (31.1 [range, 16.0 to 39.0]) (see Fig. S1 in the supplemental material). However, 9 (82%) of the 11 PCR-positive isolates with an HA titer of 2 and 21 (22%) of the 94 PCR-positive isolates with an HA titer of 0 had C_T values that fell within the range of the positive controls. These data indicate that a substantial number of A(H3) virus isolates replicated well *in vitro* and were HA negative or displayed low HA titers because they failed to efficiently agglutinate erythrocytes.

Detection of influenza A virus by NP-ELISA in tissue culture. In order to compare more directly the virus replicative capacity and hemagglutination activity, we developed an ELISA to detect viral NP in 96-well tissue culture plates. Figure 1 shows NP signals (OD values) of MDCK cultures that had been inoculated with limiting infectious doses of A/Vic/361/11 virus per well; with virus stock diluted $1:10^8$ and $1:10^9$, six and one out of eight wells, respectively, were NP positive 6 days postinoculation (dpi 6). The mean $OD_{450\text{-}620}$ value of the positive wells increased from ~1.0 at 1 dpi to >2.5 at 6 dpi. With a 10-fold-higher dose for inoculation ($1:10^7$ dilution), all eight wells became positive, with mean signals approaching the maximum $OD_{450\text{-}620}$ level of 3.0 already at 1 dpi. These signals did not decline up to 6 dpi, indicating that the viral NP remained attached to the wells for days, even after viral CPE resulted in complete elimination of the cells. Signals (mean ± standard error of the mean [SEM]) in the wells with uninfected control cells were 0.251 ± 0.002 and 0.270 ± 0.003 at 1 and 2 dpi, respectively, which declined to 0.007 ± 0.004 at 6 dpi. These results are representative of all influenza A viruses that were tested, although the kinetics and levels varied among different strains (data not shown). Due to the stability of the NP signal and the favorable signal-to-noise ratio, the detection of NP might provide a reliable alternative for detecting HA activity in culture supernatants for scoring virus-positive and virus-negative cultures in standard virus titration assays.

Comparison of HA and NP readouts for assessing infectious virus titers. To validate the NP-ELISA as an alternative readout in virus titrations, we performed three independent experiments with a panel of virus isolates ($n = 20$) with low-MDCK-passage histories and the laboratory virus strains A/Bris/059/07 (H1N1) and A/Vic/210/09 (H3N2) as controls (Table 2). Virus titers based on the detection of HA activity in the day-6 culture supernatant or NP in the cognate culture plates were similar (i.e., within 1 order of magnitude) for the two laboratory strains, as well as for the five 2008-2009 influenza A(H3) virus isolates, with ≥4.00 log $TCID_{50}$/ml and an HA titer of 128 after one *in vitro* passage. One of the 2008-2009 isolates (no. 5) had a lower infectious virus titer based on both HA and NP readouts, with ~0.75 and 2.33 log $TCID_{50}$/ml, respectively. The virus titers obtained with the HA and NP-ELISA readouts were similar for all four A(H1N1) isolates tested, including A(H1N1)pdm09, and ranged between 5.17 and 7.08 log $TCID_{50}$/ml.

In contrast, A(H3N2) viruses isolated ($n = 7$) between July 2009 and January 2011 had lower HA titers, and virus titers based on the HA readout were generally lower than those determined with the NP-ELISA. Strikingly, two virus isolates (no. 7 and 8), with mean virus titers of 6.33 log $TCID_{50}$/ml based on the NP-ELISA readout, repeatedly scored negative when HA activity was used as the readout. Using PCR we confirmed that virus actually was present in the supernatants of the NP-positive cultures (data not shown). Collectively, these results indicate that the poor capacity of these virus isolates to agglutinate RBCs precluded reli-

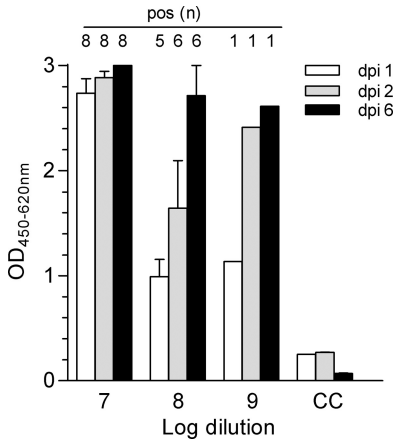

FIG 1 NP signals increase up to day 6 postinoculation. MDCK cells were inoculated with serial dilutions of A/Vic/361/11 in 96-well plates (eight replicates per dilution). On days 1, 2, and 6, NP signals were determined by ELISA. The bars indicate the $OD_{450-620}$ values (mean ± SEM) of positive wells (here defined as >2-fold above the mean of mock-inoculated cell control [CC] wells) for the 7-, 8-, and 9-log diluted virus stock. The numbers of positive (pos) wells are shown above each bar. The CC bars show the $OD_{450\text{-}620}$ values (mean ± SEM) of the eight cell control wells.

TABLE 2 Virus titers of isolates and laboratory virus strains determined by HA and NP readouts

Sample information					Results (log $TCID_{50}$/ml) by assay and expt no.										
			MDCK passage(s) of virus stocks in virus titration expts		HA					NP-ELISA					Means have <1 log difference?
ID	Influenza subtype	Date (mo/yr)	No.	HA titer	Expt 1	Expt 2	Expt 3	Mean	SD	Expt 1	Expt 2	Expt 3	Mean	SD	
1	A(H3)	Feb/2009	1	128	3.75	4.25	NT[b]	4.00	0.35	3.75	4.25	NT	4.00	0.35	Yes
2	A(H3)	Jan/2009	1	128	3.25	3.50	NT	3.38	0.18	3.75	4.25	NT	4.00	0.35	Yes
3	A(H3)	Jan/2009	1	128	5.00	5.00	NT	5.00	0.00	5.50	6.00	NT	5.75	0.35	Yes
4	A(H3)	Jan/2009	1	128	5.50	5.50	5.50	5.50	0.00	6.00	5.75	6.00	5.92	0.14	Yes
5	A(H3)	Jan/2009	1	128	<0.75	<0.75	0.75	0.75		3.00	1.25	2.75	2.33	0.95	No
6	A(H3)	Jan/2009	1	128	6.50	6.50	7.00	6.67	0.29	6.50	6.50	7.25	6.75	0.43	Yes
7	A(H3)	Jul/2009	1	2	<0.75	<0.75	<0.75	<0.75		6.25	6.00	6.75	6.33	0.38	No
8	A(H3)	Apr/2010	1	0	<0.75	<0.75	<0.75	<0.75		6.50	5.75	6.75	6.33	0.52	No
9	A(H3)	Dec/2010	2	32	3.50	2.25	2.00	2.58	0.80	3.50	4.00	4.00	3.83	0.29	No
10	A(H3)	Jan/2011	4	4	1.75	1.50	<0.75	1.63	0.18	2.50	3.00	2.75	2.75	0.25	No
11	A(H3)	Jan/2011	2	8	5.25	4.50	4.75	4.83	0.38	5.75	5.50	4.75	5.33	0.52	Yes
12	A(H3)	Jan/2011	4	8	0.75	<0.75	0.75	0.75	0.00	1.50	<0.75	1.25	1.38	0.18	Yes
13	A(H3)	Jan/2011	2	8	4.00	3.50	3.75	3.75	0.25	4.25	3.50	4.25	4.00	0.43	Yes
14	A(H1N1)	Jan/2009	2	32	5.25	5.00	5.50	5.25	0.25	5.00	5.00	5.50	5.17	0.29	Yes
15	A(H1N1)	Aug/2009	2	32	5.25	5.25	5.25	5.25	0.00	5.25	5.25	5.25	5.25	0.00	Yes
16	A(H1N1)pdm09	Jan/2011	2	>32	6.75	7.25	7.25	7.08	0.29	6.75	7.25	7.25	7.08	0.29	Yes
17	A(H1N1)pdm09	Jan/2011	2	>32	7.50	6.75	6.75	7.00	0.43	7.50	6.75	6.75	7.00	0.43	Yes
18	B	Feb/2009	1	128	3.75	3.50	3.50	3.58	0.14	<0.75	<0.75	<0.75	<0.75		No
19	B	Mar/2011	1	64	6.00	5.75	5.75	5.83	0.14	<0.75	<0.75	<0.75	<0.75		No
20	B	Jan/2011	1	>32	5.75	5.50	5.50	5.58	0.14	<0.75	<0.75	<0.75	<0.75		No
	A/Bris/059/07 A(H1N1)	NA[a]	NA	NA	5.75	5.75	5.75	5.75	0.00	5.75	5.75	5.75	5.75	0.00	Yes
	A/Vic/210/09 A(H3N2)	NA	NA	NA	5.75	5.25	4.75	5.25	0.50	5.75	5.25	4.75	5.25	0.50	Yes

[a] NA, not applicable.
[b] NT, not tested.

able virus detection and assessment of infectious virus titers. As expected, the titers of the three influenza B virus isolates no. 18 to 20) were determined by HA assay but not by NP-ELISA, because the detecting antibody used is specific for influenza A virus NP.

Performance of the NP-ELISA as readout for the assessment of infectious virus titers in clinical samples. Next, we used the NP-ELISA as a readout for determining virus titers in original clinical specimens obtained in the 2011-2012 influenza season and compared the results with those obtained using the HA activities of the culture supernatants. The samples were submitted for routine diagnostic testing for the presence of respiratory viruses ($n = 14$; see Table S1 in the supplemental material) or collected during a clinical trial designed to evaluate antiviral treatment regimens ($n = 11$; see Table S2 in the supplemental material) between January and May 2012. We tested influenza A(H3) virus PCR-positive samples only, since A(H1) virus titers were readily determined using the HA assay, which gave identical results to those of the NP-ELISA as a readout for all A(H1) titrations performed to that point (Table 2 and data not shown).

Thirteen out of the 14 original clinical specimens and eight out of the 11 clinical trial samples tested positive in the infectious virus titration with the NP readout, with mean ± SD titers of 3.23 ± 0.93 and 3.38 ± 1.08 log $TCID_{50}$/ml, respectively. In contrast, no virus was detected by the HA assay in the supernatants of the majority of NP-positive wells, which therefore produced false-negative results for 20 of the samples (<0.75 log $TCID_{50}$/ml) or lower titers (1.00 log $TCID_{50}$/ml; $n = 1$). One out of 14 clinical samples and three out of 11 trial samples tested negative also in the NP-ELISA, indicating that infectious virus titers were below the limit of detection of the virus titration assay. This correlated with the relatively high corresponding C_T values of these specimens.

DISCUSSION

Virological surveillance of influenza virus in The Netherlands showed that the frequency of A(H3) virus isolates displaying low HA activity increased between 1999 and 2012. Since their introduction in the human population in 1968, A(H3) influenza viruses have evolved continuously. Amino acid substitutions in the HA molecules affected their antigenic properties, *in vitro* replication, and capacity to agglutinate RBCs (2–6, 15–19). A subset of the 2011-2012 A(H3) isolates that lacked HA activity most likely replicated poorly *in vitro*, as evidenced by high PCR C_T values. However, low virus titers do not explain the absence of HA activity of many other A(H3) virus isolates. Up to 2008, turkey RBCs were successfully used as a readout in virus titration assays that were using hemagglutination as a readout. During influenza seasons 2009 to 2010 and 2010 to 2011, A(H1N1)pdm09 viruses were dominant. A(H3) viruses were isolated sporadically, and these isolates poorly agglutinated turkey RBCs. Influenza A(H3) viruses with poor HA activity continued to circulate and became the dominant subtype again in the 2011-2012 influenza season. The use of RBCs from other species, including guinea pigs and humans, did not consistently improve HA activity for monitoring antigenic drift by HI assays (data not shown).

The inefficient hemagglutination activity of influenza virus strains also forms a major problem for the HA readout of quantitative virus cultures of primary specimens in clinical/research settings, as well as in clinical trials. Hemagglutination has long been the preferred readout method because it is generally a simple, fast, and reliable virus detection method. It is instrumental for use as a high-throughput readout method to provide crucial information on the duration of infectious virus shedding and the ability to reduce transmission in efficacy studies of novel antiviral compounds. However, the inefficiency of virus in primary clinical specimens to agglutinate erythrocytes after the first passage in MDCK cells precluded the assessment of infectious influenza virus titers by HA readout in the majority of cases during the 2011-2012 and 2012-2013 influenza seasons, when A(H3) virus strains were dominant.

In order to equip the diagnostic laboratories with a robust HA-independent technique for the detection of progeny virus production in primary quantitative virus cultures, we employed an ELISA that measures NP in multiwell virus culture plates. This NP-ELISA is based on the method described for the WHO-recommended microneutralization assay (8, 9). One important difference with virus titration assays is that in neutralization assays, cell cultures are inoculated with 100× $TCID_{50}$/well of known virus strains after preincubation with serial serum dilutions, and virus production is monitored 1 day after inoculation. To propagate and quantify viruses present in original clinical specimens, other plate types are used, and an incubation period of 6 days is recommended to allow for the detection of slowly replicating viruses in wells around the endpoint dilution. Therefore, we evaluated the stability of the NP signal up to 6 days after inoculation and showed that it increased or remained stable but never declined. In wells around the endpoint dilution, signals generally reached levels that were comparable to those achieved with a 10- to 100-fold-higher multiplicity of infection.

With HA-competent viruses, the HA and NP readout methods detected virus replication in largely the same wells and resulted in similar titers. In contrast, the infectious virus titers of virus isolates and clinical samples containing HA-deficient viruses were determined only by the NP-ELISA. The HA assay may still be preferred as a first readout method, since it requires less time and fewer reagents, and it detects A(H1N1), A(H1N1)pdm09, and influenza B viruses in the culture supernatants. The tissue culture plates used for determining infectious virus titers can be stored at −20°C until the subtype of the influenza A viruses is identified by PCR, allowing for the detection of virus replication by NP-ELISA as a second readout method in the same titration experiment. The detection of NP relies on the binding of the NP-specific monoclonal antibody HB65 that reacts with a broad range of influenza A viruses (13, 14, 20), including the A(H1N1) viruses from 1934 (A/PR/8/34) and A(H3N2) and A(H1N1)pdm09 viruses isolated in 2012, as reported here. Other recent viruses that were recognized include A/Vic/361/11 (H3N2) and A/Cal/07/09 (H1N1), and also avian and swine influenza viruses A(H5N1), A(H7N7) (14), and A(H7N9) (21). The potential emergence of variants that are not recognized by monoclonal antibody HB65 should be carefully monitored, but based on the documented conservedness of the epitope that is recognized, this is not expected to occur anytime soon. Alternatively, a cocktail of monoclonal antibodies can be used to minimize this risk even further.

We have tested a number of influenza B virus NP-specific antibody preparations to evaluate the ELISA-based detection of influenza B virus-infected cultures at day 6 after inoculation. Although several preparations recognized all Victoria and Yamagata lineage strains tested, in many cases, signals were too low to distinguish positive and negative wells in a robust way (data not shown). Because current influenza B viruses do agglutinate erythrocytes efficiently, there is no urgent need for alternative readout methods.

The antigenic characterization of virus isolates by HI assays forms the basis for the annual selection of vaccine strains (15, 16). Due to the reduced capacity of A(H3N2) viruses to agglutinate chicken RBCs and, more recently, turkey RBCs, HI data are currently also generated using mammalian RBCs from guinea pigs and humans. However, agglutination patterns are generally more difficult to read, and turkey RBCs are still frequently used for antigenic characterization, possibly leading to an underrepresentation of circulating A(H3N2) virus variants for antigenic characterization. Because of the factors that complicate the HI assay, including the need to consider RBCs from different species for different virus subtypes, virus-neutralizing (VN) assays are now also used for antigenic characterization (22–24) and for evaluations of vaccines with relatively low immunogenicity in naive human populations, e.g., H5 and H7 (8, 25, 26). The ELISA method used in such VN assays detects viruses irrespective of their capacity to agglutinate RBCs, a feature that was successfully adapted to improve the monitoring of infectious virus titers in specimens of clinical trials for antivirals.

In conclusion, we provide epidemiological data on the emergence of A(H3) influenza viruses that fail to efficiently agglutinate erythrocytes. The HA deficiency of these viruses complicates the monitoring of virus replication in quantitative virus culture and assessment of infectious virus titers in clinical specimens. In order to provide for an HA-independent readout for quantitative virus cultures of primary patient samples in clinical/research and clinical trial settings, we validated the use of NP detection by ELISA on day six postinoculation of MDCK cells. For currently circulating A(H3) viruses, the HA readout yielded mainly false-negative results, while the NP readout proved to be a robust method that facilitates the measurement of virus titers in clinical specimens, which is critical for the evaluation of new antiviral compounds and therapies in clinical trials.

ACKNOWLEDGMENTS

We thank Roche for sharing data on virus titrations of clinical trial specimens.

C.A.V.B., C.E., and L.S. are employed by Erasmus MC spin-out company Viroclinics Biosciences BV, for which A.D.M.E.O. and G.F.R. are consultants.

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